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RESEARCH ARTICLE

# An ancestral non-proteolytic role for presenilin proteins in multicellular development of the social amoeba *Dictyostelium discoideum*

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## ABSTRACT

Mutations in either of two presenilin genes can cause familial Alzheimer's disease. Presenilins have both proteolysis-dependent functions, as components of the γ-secretase complex, and proteolysis-independent functions in signalling. In this study, we investigate a conserved function of human presenilins in the development of the simple model organism *Dictyostelium discoideum*. We show that the block in *Dictyostelium* development caused by the ablation of both *Dictyostelium* presenilins is rescued by the expression of human presenilin 1, restoring the terminal differentiation of multiple cell types. This developmental role is independent of proteolytic activity, because the mutation of both catalytic aspartates does not affect presenilin ability to rescue development, and the ablation of nicastrin, a γ-secretase component that is crucial for proteolytic activity, does not block development. The role of presenilins during *Dictyostelium* development is therefore independent of their proteolytic activity. However, presenilin loss in *Dictyostelium* results in elevated cyclic AMP (cAMP) levels and enhanced stimulation-induced calcium release, suggesting that presenilins regulate these intracellular signalling pathways. Our data suggest that presenilin proteins perform an ancient non-proteolytic role in regulating intracellular signalling and development, and that *Dictyostelium* is a useful model for analysing human presenilin function.

**KEY WORDS: *Dictyostelium*, γ-secretase, Presenilin**

## INTRODUCTION

To date, more than 170 different mutations in presenilin 1 (*PSEN1*) and 13 mutations in presenilin 2 (*PSEN2*) genes are known to give rise to a familial form of Alzheimer's disease (FAD) (De Strooper and Annaert, 2010). The protein products of the mammalian *PSEN* genes are components of an aspartate protease complex, γ-secretase (De Strooper, 2003), which is responsible for the regulated, intramembranous cleavage of a number of type 1 transmembrane proteins (Wolfe, 2006), including the Notch receptors (Huppert et al., 2000) and the amyloid precursor protein (APP). It is from APP that β-amyloid (Aβ), the likely causative agent of both the familial and sporadic forms of Alzheimer's disease, is derived (De Strooper et al., 1998; Herreman et al., 2000). Prior to incorporation into the γ-secretase complex, the presenilin 1 protein undergoes autoproteolytic cleavage into two parts (Chávez-Gutiérrez et al., 2008). Presenilin proteins have also been shown to have a non-proteolytic function as a scaffold for the regulation of glycogen synthase kinase 3β (GSK3β)-dependent β-catenin phosphorylation (Kang et al., 1999; Kang et al., 2002). Finally, presenilin proteins have also been implicated in altered calcium signalling (Tu et al., 2006) through an unknown mechanism. Understanding the discrete roles of presenilin proteins thus remains an important goal in understanding basic cell function and the progression of Alzheimer's disease (De Strooper and Annaert, 2010).

Although research into presenilin protein function has often focussed on presenilin-knockout mice (Schaeffer et al., 2011), it has been difficult to determine the precise function of presenilins because the loss of both presenilins leads to embryonic lethality. A range of alternative non-mammalian models have been employed to examine the function of presenilin proteins, including *Physcomitrella patens* (Khandelwal et al., 2007), *Caenorhabditis elegans* (Calahorro and Ruiz-Rubio, 2011), *Danio rerio* (van Tijn et al., 2011) and *Drosophila melanogaster* (Coen et al., 2012). However, the social amoeba *Dictyostelium discoideum* is the simplest model organism that possesses two presenilin proteins as well as the other three components of the γ-secretase complex (Boeckeler and Williams, 2007; McMains et al., 2010). *Dictyostelium* has been extensively used in a range of motility (Janetopoulos and Firtel, 2008), developmental (Loomis and Shaulsky, 2011) and biomedical studies (Chang et al., 2012; Francione and Fisher, 2011; Ludtmann et al., 2011; Myre et al., 2011; Terbach et al., 2011), and has a number of experimental advantages over existing models (Williams et al., 2006). In this study, we employ the advantages of *Dictyostelium* to explore the role of the human presenilin 1 protein (PSEN1) in development. We generated an isogenic *Dictyostelium* strain lacking both presenilin A and presenilin B genes (*psenA*$^{-}$/*psenB*$^{-}$), and found that development was blocked at the aggregation stage prior to morphogenesis. We show that either human PSEN1 or *Dictyostelium* PsenB can rescue the developmental phenotype of this mutant. Presenilin proteins that are mutated at the catalytic aspartic acid residues retain the ability to rescue the developmental block, demonstrating that the proteolytic activity of presenilin is not required for phenotypic rescue. A conserved functional role for

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the human protein in *Dictyostelium* provides new insights into the ancient function(s) of presenilin proteins.

## RESULTS

### *Dictyostelium* presenilins share substantial similarity with human presenilins

The social amoeba *Dictyostelium discoideum* represents one of the earliest branches from the common ancestor of all eukaryotes, and thus provides a useful means for understanding the ancestral eukaryotic genome (Eichinger et al., 2005). It is also a simple widely used model organism for understanding developmental signalling and is increasingly being used to investigate the role of disease-related proteins (Williams et al., 2006). The *Dictyostelium* genome sequence has identified orthologues of a range of proteins associated with human diseases and conditions (Eichinger et al., 2005), including two presenilin proteins (PsenA and PsenB). Phylogenetic analysis reveals that they are more closely related (sister) to the monophyletic animal presenilin clade than to the distinct monophyletic plant presenilin clade (Fig. 1A). Furthermore, PsenA and PsenB (Fig. 1A) are similar in size and structure to the two human homologues and, like their human counterparts, they contain the conserved catalytic aspartic acid residues for proteolytic activity (Wolfe, 2006) and the GxDG motif for γ-secretase integration (Fig. 1B). A comparison of the predicted transmembrane domains reveals that these regions are 43–50% identical in the *Dictyostelium* and human proteins. Additionally, more than half (67/112) of the residues in either PSEN1 or PSEN2 that are mutated in FAD are conserved in either *Dictyostelium* PsenA or PsenB (supplementary material Fig. S1) [see also fig. S2 in McMains et al. (McMains et al., 2010)]. The sequence similarity and predicted common structure between the human and *Dictyostelium* presenilin proteins suggests a possible conservation of their function in these distantly related species.

### Presenilins function redundantly in development

Presenilin proteins have previously been shown to play a key role in *Dictyostelium* development (McMains et al., 2010). In wild-type cells, starvation triggers the aggregation of ~100,000 individual amoebae that progress through a series of multicellular morphological stages over a 24-h period, ultimately giving rise to a fruiting body comprising spores held within a sorus suspended above the substratum by a stalk composed of dead vacuolated cells (Fig. 2) (Williams et al., 2006). To test whether PsenA and PsenB act redundantly, we ablated each *Dictyostelium* gene individually using homologous integration of a knockout cassette (Faix et al., 2004) (supplementary material Fig. S2). The ablation of either *Dictyostelium* gene individually did not alter this basic developmental morphology under our conditions (supplementary material Fig. S3A), in contrast to earlier reports showing a developmental block following *psenB* ablation (McMains et al., 2010), which might be accounted for by a difference in parental strain or by differing experimental conditions. However, the removal of both genes (generating a line hereafter referred to as *psenA*$^{-}$/*psenB*$^{-}$ cells) caused a strong block in development (Fig. 2). The *psenA*$^{-}$/*psenB*$^{-}$ cells aggregated to complete early development, forming a rounded structure attached to the substratum, but morphogenesis halted at the beginning of tip formation. This supports the role previously suggested for *Dictyostelium* presenilins in development (McMains et al., 2010) and demonstrates that the presenilin homologues have redundant functions, consistent with what is observed in mammals (Feng et al., 2004; Kim et al., 2011).

### Human PSEN1 rescues the defective development of *psenA*$^{-}$/*psenB*$^{-}$ cells

To investigate whether *Dictyostelium* and human presenilin proteins have conserved functions, we expressed the PsenB and PSEN1 proteins tagged with an N-terminal GFP (Veltman et al., 2009) in the *psenA*$^{-}$/*psenB*$^{-}$ genetic background. Expression of either PsenB or PSEN1 reversed the morphological block in development in this mutant (Fig. 2), resulting in the formation of wild-type fruiting bodies composed of both a stalk and a fully formed sorus (Fig. 2). We also expressed the human PSEN2 protein tagged with an N-terminal GFP, and found that development was partially rescued, so that it progressed beyond the block at the tipped mound observed in *psenA*$^{-}$/*psenB*$^{-}$ cells, to the early culminant (Fig. 2). Because PSEN2 expression did not fully complement development in the *psenA*$^{-}$/*psenB*$^{-}$ genetic background, our subsequent analyses were restricted to human

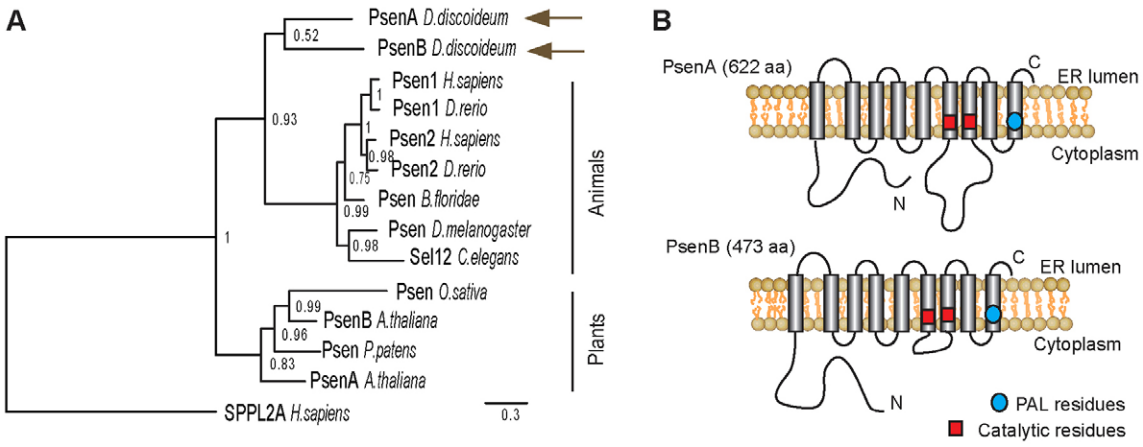


**Fig. 1. The *Dictyostelium* PsenA and PsenB proteins show structural similarity to human presenilins.** (A) Bayesian-derived phylogeny of presenilin amino-acid-sequence data from animal and plant species and *D. discoideum*. Human SPPLA2A was used as the outgroup. Numbers adjacent to nodes represent Bayesian posterior probability values. Scale bar: the number of substitutions per site. (B) Schematic of both *Dictyostelium* presenilin proteins showing the putative orientation in the membrane, cytosolic and transmembrane regions, the conserved catalytic residues and the proline-alanine-leucine (PAL) sequence.

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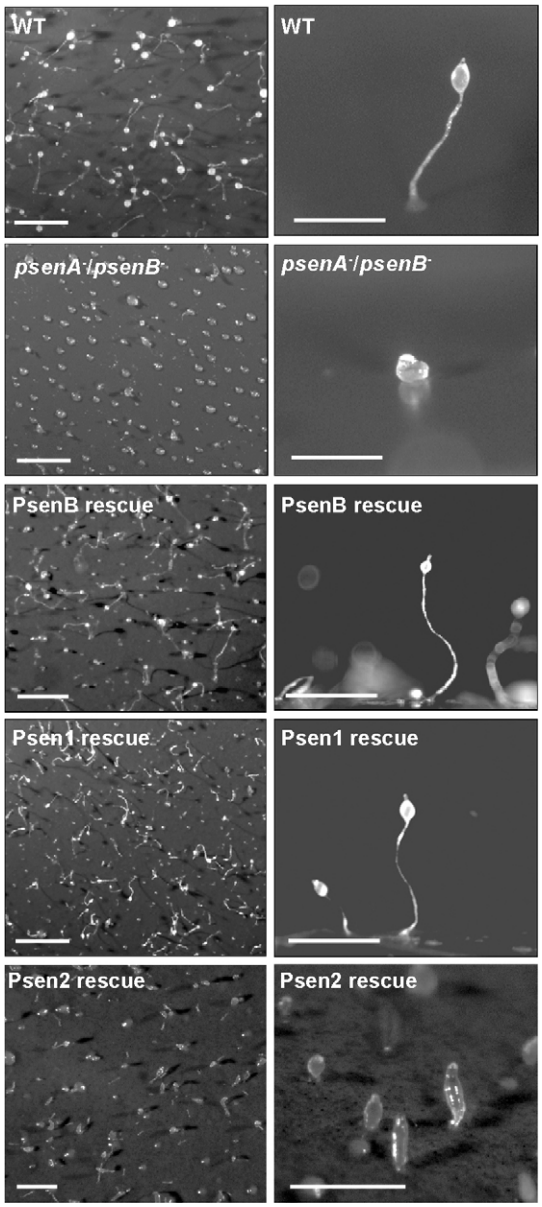


**Fig. 2. *Dictyostelium* presenilin proteins redundantly control multicellular development that is complemented by expression of human presenilin 1.** Following starvation, wild-type (WT) cells undergo development over a 24-h period, leading to the formation of fruiting bodies; left, aerial view at low magnification; right, side view at higher magnification. Ablation of both presenilin genes (*psenA*$^{-}$/*psenB*$^{-}$) in one cell line gives rise to a block in fruiting-body formation, leading to a small round structure lacking a stalk. Overexpression of GFP-tagged *Dictyostelium* PsenB or human PSEN1 in these cells restores development, whereas PSEN2 expression partially rescues development. Scale bars: 1 mm.

PSEN1. This developmental complementation by PSEN1 in this model organism suggests that the function of PSEN1 is conserved from humans to *Dictyostelium*.

Our previous study also identified altered development in *Dictyostelium* lacking presenilin activity (McMains et al., 2010), so we assessed whether the *Dictyostelium* and human presenilin proteins could complement these defects in stalk- and spore-cell production and developmental-marker expression (Fig. 3). Stalk-cell formation was induced in low-density cultures in the presence of differentiation induction factor-1 (DIF-1) (Fig. 3A) (Williams et al., 1999). The ablation of either *psenA* or *psenB* had no effect on DIF-1-induced stalk-cell formation, whereas the *psenA*$^{-}$/*psenB*$^{-}$ mutant, although still able to form stalk cells, showed a significant 31% reduction in stalk-cell production ($P<0.05$), mirroring the developmental phenotype of each mutant. We also analysed the inhibition of this developmental pathway by cAMP, which leads to a glycogen-synthase-kinase-A (GSKA)-dependent block in stalk-cell production (GSKA is the *Dictyostelium* homologue of mammalian GSK3β) (Williams et al., 1999). The ablation of either *Dictyostelium* presenilin gene had no effect on cAMP-mediated inhibition of stalk-cell differentiation compared with wild-type cells, whereas the presenilin double-null mutant showed a significant 84% increase in cAMP-dependent stalk-cell inhibition compared with wild-type cells ($P<0.004$), which is consistent with the absence of a stalk in the fruiting body after 24 h. Furthermore, complementing the *psenA*$^{-}$/*psenB*$^{-}$ mutant with PsenB and PSEN1 restored stalk-cell production to levels close to that achieved by wild-type cells either in the presence or absence of cAMP.

Because *Dictyostelium* presenilin proteins have previously been shown to regulate spore production in monolayer assays (McMains et al., 2010), we also assessed whether expressing the *Dictyostelium* and human proteins complemented this aspect of multicellular development (Fig. 3B). The ablation of either *psenA* or *psenB* caused a 40% reduction in spore production during multicellular development. However, the *psenA*$^{-}$/*psenB*$^{-}$ mutant showed a complete loss of spore formation. The expression of either PsenB or PSEN1 restored spore production to the level of single knockouts, confirming the functionality of the human presenilin protein in *Dictyostelium* development. Together with the effects on stalk-cell differentiation and the observed developmental arrest at the initiation of tip formation, our results indicate that presenilin proteins are required not for cell-type choice but for differentiation along both the stalk and spore pathways.

Finally, as an independent verification of developmental complementation, we used quantitative transcriptional analysis (qPCR) to monitor the expression of developmental and cell-type markers in the *psenA*$^{-}$/*psenB*$^{-}$ mutant and following complementation with the *Dictyostelium* and human proteins (Fig. 3C). The cAMP receptor 1 (*cAR1*) gene is expressed in wild-type cells in early development (Saxe, III et al., 1991), whereas a threefold increase in expression in early (6 h) development and elevated expression in mid–late (12–24 h) development was observed in the *psenA*$^{-}$/*psenB*$^{-}$ mutant compared with wild-type cells. In addition, the pre-stalk-cell marker *ecmA* (Harwood, 2008) showed a highly significant reduction in expression in the *psenA*$^{-}$/*psenB*$^{-}$ mutant in early and mid-development, whereas the pre-spore-cell marker *psA* (Harwood, 2008) showed only a small elevation in the double mutant compared with wild-type cells after 24 h of development. Complementation with PsenB returned marker expression to wild-type levels. Complementation with PSEN1 only partially rescued marker expression, with levels not reaching those of either wild-type cells or *psenA*$^{-}$/*psenB*$^{-}$ cells complemented with PsenB, suggesting a reduced efficacy of the human protein in *Dictyostelium*. Also, expression of either human or *Dictyostelium* proteins caused an elevation of late (24 h) stalk-cell-marker expression and mid-late (12–24 h) spore-cell-marker expression, suggesting a common controlling role for both presenilin proteins in terminal differentiation. These data suggest that the

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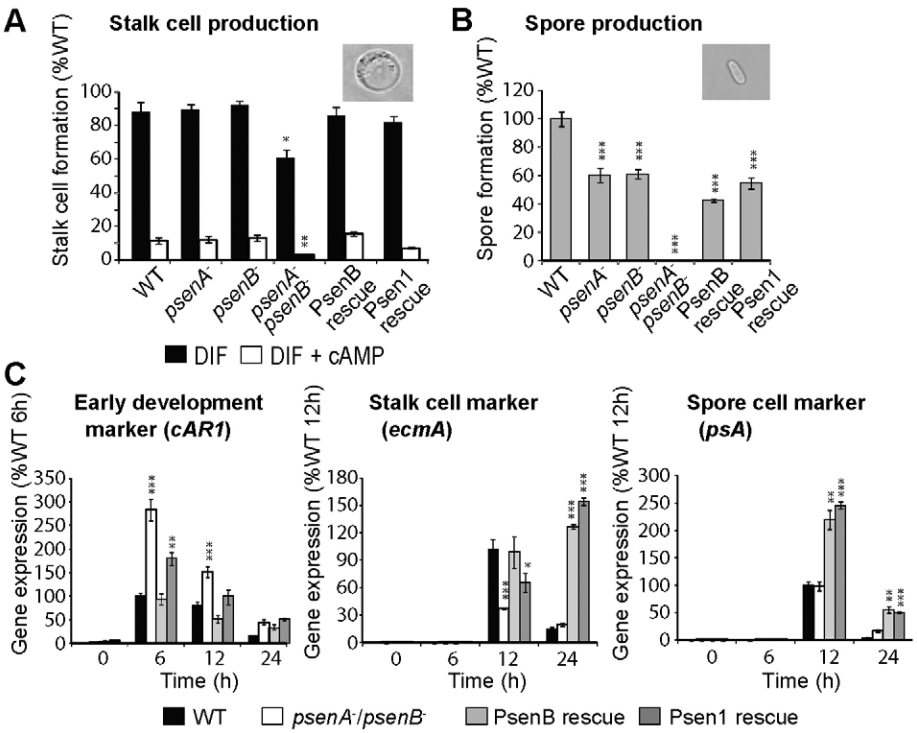


**Fig. 3. Expression of PsenB or PSEN1 can rescue the block in multiple differentiation pathways resulting from the ablation of presenilin activity in the *psenA*$^{-}$/*psenB*$^{-}$ mutant.** (A) Stalk-cell production is unaffected by ablation of either presenilin individually but is reduced when both presenilins are removed and is restored by expression of PsenB or PSEN1. (B) Spore production is reduced by ablation of either presenilin gene individually, is blocked in *psenA*$^{-}$/*psenB*$^{-}$ mutants and is restored by expression of PsenB or PSEN1. Insets show light microscopy images of the cell type analysed in each panel. (C) The expression of developmentally regulated genes (0, 6, 12 and 24 h post-starvation), using quantitative transcriptional analysis for the early developmental marker *cAR1* and the cell-type-specific marker genes (pre-stalk, *ecmA*; pre-spore, *psA*) in wild-type and in *psenA*$^{-}$/*psenB*$^{-}$ cells, and following complementation with PsenB or PSEN1. Increased *cAR1* expression and reduced *ecmA* expression caused by the loss of both presenilin genes is restored by the expression of PsenB or PSEN1. All data are presented as the mean ± standard deviation. **P*<0.05, ***P*<0.01, ****P*<0.001.

differentiation of pre-stalk and pre-spore cells in the mound preceding tip formation is not dramatically impaired by the loss of presenilin (but is enhanced by the ectopic expression of either the *Dictyostelium* or human presenilin protein). By contrast, the further differentiation to mature stalk and spore cells is blocked, as is morphogenesis beyond the beginning of tip formation. These data also confirm the functionality of the human presenilin 1 protein in stalk- and spore-cell differentiation in *Dictyostelium*.

### The presenilins are predominantly localised at the endoplasmic reticulum in *Dictyostelium*

We next examined the subcellular localisation of the expressed *Dictyostelium* and human proteins in the *psenA*$^{-}$/*psenB*$^{-}$ mutant. In mammals, presenilins are localised on the endoplasmic reticulum (ER) (Walter et al., 1996) and nuclear membrane (Li et al., 1997). In *Dictyostelium*, presenilin subcellular localisation was determined by fixing cells expressing GFP-tagged versions of PsenB and PSEN1 to reveal that both proteins were located on a fine interlaced network of membrane structures resembling the ER and nuclear envelope (Fig. 4). Colocalisation with an ER-specific marker, calnexin (Müller-Taubenberger et al., 2001), confirmed that both the human and *Dictyostelium* presenilin proteins localise to the ER and nuclear envelope in *D. discoideum*. These data once again confirm the similar behaviour of human and *Dictyostelium* presenilin proteins.

### The proteolytic activity of presenilins is dispensable for development

We next investigated the molecular mechanism of presenilin function in *Dictyostelium*. The human presenilin proteins have been ascribed either a proteolytic function within the γ-secretase complex, dependent upon two crucial catalytic aspartic-acid residues in the protein (Yamasaki et al., 2006), or a structural role related to the docking of intracellular signalling components (Fluhrer et al., 2004; Tesco and Tanzi, 2000; Twomey and McCarthy, 2006). The *Dictyostelium* presenilin proteins have been shown to proteolytically process recombinant human APP in an identical manner to APP processing in mammalian cells (McMains et al., 2010). This is probably due to γ-secretase activity because the proteolytic ability is lost upon ablation of the γ-secretase components nicastrin (Ncstn) and anterior pharynx defective 1 (Aph1) (McMains et al., 2010). To establish a role for γ-secretase proteolytic activity in *Dictyostelium* development, we mutated the catalytic aspartic-acid residues to alanines in both *Dictyostelium* and human proteins [D348A/D394A in PsenB or D257A/D385A in PSEN1 (Tesco and Tanzi, 2000); see supplementary material Fig. S1] and examined whether these mutant presenilin proteins could complement the defective fruiting-body formation of *psenA*$^{-}$/*psenB*$^{-}$ cells. Expression of either the *Dictyostelium* or human double aspartic-acid-mutated protein rescued *Dictyostelium* morphological development, enabling wild-type fruiting bodies to form (Fig. 5A). Furthermore, ablation of the *ncstn* gene, which is necessary for γ-secretase proteolytic ability (Edbauer et al., 2003), had no effect on fruiting-body development (Fig. 5A). As an independent verification of the normal developmental morphology that we observed in these experiments, we measured spore production in the complemented *psenA*$^{-}$/*psenB*$^{-}$ cells or the *ncstn*$^{-}$ mutant and confirmed that spore production was similar to the wild-type cells (Fig. 5B). These results suggest that presenilin proteins do not require proteolytic activity, presumably as part of the γ-secretase complex, to function in *Dictyostelium* development.

The proteolytic activity of mammalian presenilins depends on their PEN2-dependent autoproteolytic cleavage at the intracellular loop, giving rise to a 34-kDa N-terminal and a 22-kDa C-terminal fragment (CTF) (Ahn et al., 2010; Haass and De Strooper, 1999). We therefore probed protein extracts from *psenA*$^{-}$/*psenB*$^{-}$ cells transformed with GFP-tagged wild-type PSEN1 and PSEN2 by western blotting, to ascertain whether

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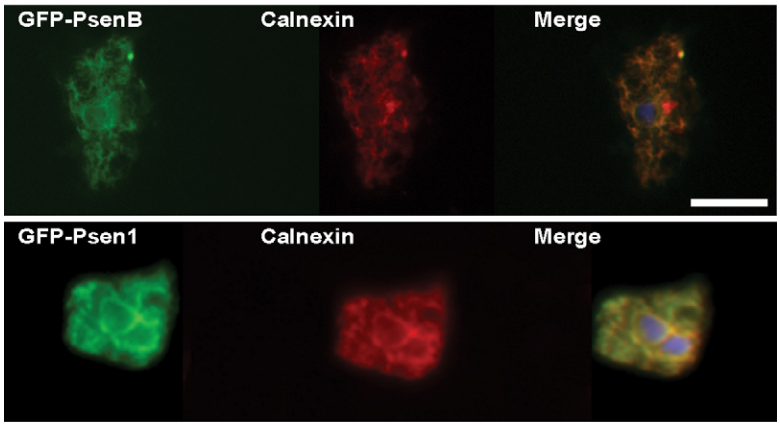


**Fig. 4. The *Dictyostelium* and human presenilins localise to the ER in *Dictyostelium* cells.** The GFP tag on PsenB and PSEN1 proteins indicates that both proteins colocalise with the ER marker calnexin (labelled with a specific antibody). Blue, 4′,6-diamidino-2-phenylindole (DAPI) staining. Scale bar: 10 µm.

cleavage occurred. This analysis showed that a band of 20–25 kDa corresponding to the CTF was detected for both proteins (Fig. 5C), indicating that endoproteolysis of both the human presenilins occurs in *Dictyostelium*. This result suggests that these human proteins can form an active protease complex together with the remaining *Dictyostelium* γ-secretase components.

### Presenilins function in cAMP signalling and calcium homeostasis in *Dictyostelium*

We then investigated two signalling pathways that might be controlled by presenilin activity during *Dictyostelium* development. A role for presenilins in cAMP signalling was likely, based upon two observations: first, *psenA*$^{-}$/*psenB*$^{-}$ cells do not show streaming during early development (where a visible trail of aggregating cells is seen leading to a mound; supplementary material Movie 1 and supplementary material Fig. S3B) – a phenotype associated with altered cAMP signalling (Garcia and Parent, 2008; Veltman and van Haastert, 2008); and second, the mutant cells also show increased sensitivity to cAMP-induced stalk-cell inhibition in late development (Fig. 3A). Therefore, we assessed intracellular cAMP levels during early development in wild-type cells, the *psenA*$^{-}$/*psenB*$^{-}$ mutant and double-mutant cells complemented with PsenB or PSEN1 (Fig. 6A). No significant difference was found between the different strains during growth ($t$=0 h) or early development ($t$=4 h). However, at $t$=8 h, the *psenA*$^{-}$/*psenB*$^{-}$ mutant showed significantly higher cAMP levels than wild-type cells ($P$<0.001). This was rescued following complementation with PsenB and

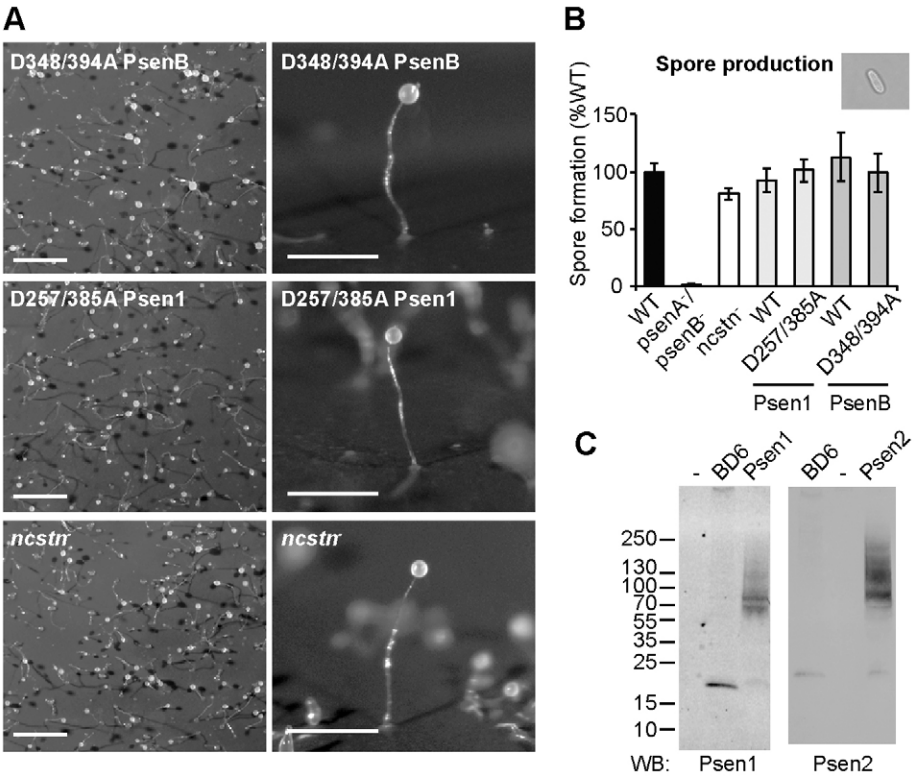


**Fig. 5. *Dictyostelium* fruiting-body development does not require the proteolytic activity of presenilins or a complete γ-secretase complex.** (A) Expression of *Dictyostelium* PsenB$^{D348A/D394A}$ or human PSEN1$^{D257A/D385A}$, lacking the key catalytic aspartic-acid residues that are necessary for proteolytic activity, rescued *Dictyostelium* development in *psenA*$^{-}$/*psenB*$^{-}$ cells. The ablation of nicastrin (*ncstn*$^{-}$) had no effect on *Dictyostelium* fruiting-body (developmental) morphology. Images show low-magnification aerial view (left) and high-magnification side view (right). Scale bars: 1mm. (B) Spore production in *psenA*$^{-}$/*psenB*$^{-}$ cells is restored by expression of either *Dictyostelium* or human presenilins mutated at the two catalytic aspartic-acid residues and is unaffected by the ablation of nicastrin (*ncstn*$^{-}$). WT, wild type. Results show means±s.e.m. Inset shows a light microscopy image of the cell type analysed. (C) Both human presenilins, when expressed in *Dictyostelium* *psenA*$^{-}$/*psenB*$^{-}$ cells, undergo endoproteolysis to yield a single band of 20–25 kDa, corresponding to the C-terminal fragment, as demonstrated by western analysis. Molecular-mass markers in kilodaltons are indicated on the left; -, untransformed *psenA*$^{-}$/*psenB*$^{-}$ control; BD6, mouse-blastocyst-like cells showing processing of endogenous mouse presenilins. The antibodies used for western blotting (WB) are indicated below the blots.

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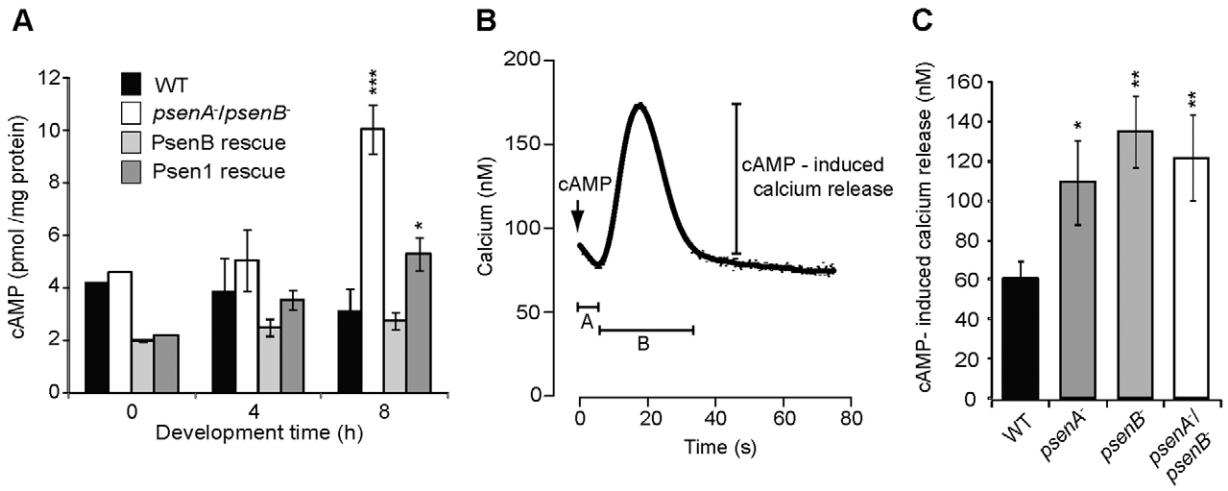


**Fig. 6. Loss of presenilin proteins in *Dictyostelium* alters cAMP and calcium levels.** (A) cAMP levels in cells during growth ($t$=0 h) and early development ($t$=4 and 8 h) in wild-type (WT) cells and *psenA*$^{-}$/*psenB*$^{-}$ cells with and without complementation (rescue) with PsenB or PSEN1. Data are derived from five independent experiments and show a statistically significant increase in cAMP in *psenA*$^{-}$/*psenB*$^{-}$ and the PSEN1-rescue cells at 8 h only. (B) Schematic representation of the calcium-response recording prior to and upon 50 nM cAMP stimulation; A, the time until calcium response after cAMP stimulation; B, the length of the calcium response. The magnitude of the cAMP-induced calcium release is also shown. Data for A and B are presented in supplementary material Fig. S4. (C) The magnitude of the calcium response in wild-type and presenilin-mutant cells. The magnitude of the calcium response is significantly increased in *psenA*$^{-}$, *psenB*$^{-}$ and *psenA*$^{-}$/*psenB*$^{-}$ cells when compared to wild-type cells. Values shown are means±s.e.m, $n$=5. *$P$<0.05, **$P$<0.01, ***$P$<0.001.

reduced following complementation with PSEN1, and both proteins rescued the lack of streaming observed in early development (supplementary material Fig. S3B). These data demonstrate a role for the *Dictyostelium* presenilin proteins in regulating cAMP signalling, an effect that has also been seen in mammals (Müller et al., 2011; Wang et al., 2011). Once again, these results demonstrate that the *Dictyostelium* and human proteins have overlapping functions and perform a conserved role in *Dictyostelium* development.

Because both human and *Dictyostelium* presenilin proteins are localised to the ER and presenilin ablation has been shown to alter calcium signalling in mammals (Tu et al., 2006), we also analysed whether calcium levels were affected in the presenilin mutants. In these experiments, cells expressing apoaequorin were loaded with coelenterazine and calcium levels were measured by luminescence in resting cells and following cAMP stimulation (Fig. 6). This analysis showed that the resting calcium levels, the cAMP-dependent time until calcium response and the duration of the calcium response were not significantly different in the single- and double-presenilin-null cells when compared to wild-type cells (supplementary material Fig. S4). However, the magnitude of the cAMP-induced calcium response was 2–2.5-fold greater in *psenA*$^{-}$, *psenB*$^{-}$ and *psenA*$^{-}$/*psenB*$^{-}$ cells ($P$<0.05, $P$<0.01 and $P$<0.01, respectively) when compared to the wild-type cells (Fig. 6C). These results suggest a role for presenilin proteins in *Dictyostelium* calcium homeostasis.

### *Dictyostelium* presenilins can cleave mammalian substrates

The conserved function for the human presenilin 1 protein in *Dictyostelium* development prompted us to examine whether the reverse is true; that is, whether *Dictyostelium* presenilins can function in mammalian cells. We tested the ability of presenilin proteins to function in the human γ-secretase complex to cleave Notch-1 within the transmembrane domain, as measured by the activation of a construct containing a Notch-and-CBF1-dependent luciferase reporter gene (Hooper et al., 2006). We transfected mouse-blastocyst-derived cells lacking all copies of both presenilin genes [BD8 cells (Donoviel et al., 1999)] with constructs expressing either PsenA, PsenB (both with and without FLAG tags) or human PSEN1, an S2-cleaved but membrane-tethered form of human Notch-1 (ΔEN1) and the CBF1-luc reporter construct. Both *Dictyostelium* presenilin proteins showed significant activity in their ability to participate in γ-secretase cleavage of the S3 cleavage site in ΔEN1, and to induce reporter activation (Fig. 7), indicating that the *Dictyostelium* presenilin proteins are functionally active in mammalian cells, presumably as part of the γ-secretase complex. These results are in agreement with our previous demonstration of the cleavage of an N-terminally truncated human APP in *Dictyostelium* (McMains et al., 2010).

## DISCUSSION

There is a pressing need for new models in presenilin research owing to the lethality of double-presenilin-null mutants in mammals (Shen et al., 1997), which makes research in the absence of background presenilin activity highly problematic. Alternative non-mammalian model systems (Calahorro and Ruiz-Rubio, 2011; Coen et al., 2012; Khandelwal et al., 2007; van Tijn et al., 2011) have been used to provide some insight into presenilin function, but studies in these models are complicated by difficulties in gene ablation or in the overexpression of tagged proteins in stable isogenic cell lines to enable cell-signalling and biochemistry studies. This study in *Dictyostelium*, in combination with our previous work (McMains et al., 2010), thus demonstrates the experimental benefits provided by this novel model for the analysis of presenilin function.

We have addressed the conservation of function of the *Dictyostelium* and human presenilins in development by analysing their ability to rescue the block mid-way through development (at the mound stage) observed in a *psenA*$^{-}$/*psenB*$^{-}$ mutant (Fig. 2). The ability of PSEN1 to efficiently restore morphological development strongly supports the conclusion that

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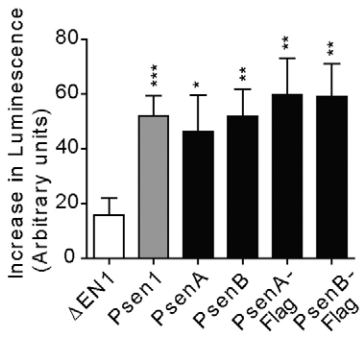


**Fig. 7. The *Dictyostelium* presenilins are functional in mammalian cells.** The PsenA and PsenB proteins were expressed in mouse-blastocyst-derived BD8 cells that have both *psen1* and *psen2* ablated. γ-secretase activity was assessed by the fold increase in luminescence resulting from cleavage of the substrate ΔEN1 (a membrane-tethered Notch1 lacking the extracellular domain) by transfected human presenilin 1 (WT) or *Dictyostelium* presenilin proteins (PsenA and PsenB) with or without a C-terminal FLAG tag. Results show means±s.e.m. Data represent two to three independent experiments carried out in duplicate ($n$=4–6). *$P$<0.05, **$P$<0.01, ***$P$<0.001.

the human and *Dictyostelium* presenilins share a conserved and evolutionarily ancient function. In addition, the ability of PsenB to rescue the development of the double-null mutant suggests that there is functional redundancy between the *Dictyostelium* presenilins, even though their starkly different expression profiles (McMains et al., 2010), with *psenB* expressed highly in growth and *psenA* expressed highly during later development, would suggest specific functions for each presenilin. The reverse is also true: APP expressed ectopically in *Dictyostelium* is cleaved efficiently by the endogenous γ-secretase complex and in a similar way as in mammalian cell lines (McMains et al., 2010), and we show that *Dictyostelium* presenilins can cleave mammalian Notch 1 when they are expressed in *Psen1*$^{-}$/*Psen2*$^{-}$ mouse-blastocyst-like cells (Fig. 7). Thus, it appears that human presenilin can be incorporated into the *Dictyostelium* γ-secretase complex and vice versa, and that both of these complexes are proteolytically active. Additionally, the shared cellular localisation [on the ER and nuclear envelope (Walter et al., 1996)] in both species and a shared function in regulating intracellular (cAMP) signalling (Müller et al., 2011; Wang et al., 2011) and calcium homeostasis (Berridge, 2011) further support a common function for presenilins in mammals and *Dictyostelium* (Figs 4,6). These observations together suggest that presenilins might have a conserved function throughout multiple kingdoms of life and across millennia of evolution.

Despite a conserved function of the *Dictyostelium* and human presenilin proteins in a range of cellular processes, the physiological function of the proteins in the development of the two species appears to be different. Mutating the catalytic aspartic-acid residues of either PsenB or PSEN1 did not remove the ability of these proteins to rescue the morphological defect of the *psenA*$^{-}$/*psenB*$^{-}$ mutant during development (Fig. 5A). Also, ablation of nicastrin, which completely blocks γ-secretase proteolytic activity in mammalian models (Edbauer et al., 2003), did not block *Dictyostelium* development, unlike the strong effect observed when both *psenA* and *psenB* are disrupted. Thus, these data showing that presenilin proteolytic activity is dispensable for development suggest an archaic structural role for presenilin proteins in this amoeba, which is independent of proteolytic function. In support of this, a study in the moss *Physcomitrella patens* showed that both human presenilin 1 and the PSEN1$^{D385A}$ variant were able to rescue the growth defect observed in the moss presenilin-null mutant (Khandelwal et al., 2007). However, it appears that certain growth and other developmental aspects in *Dictyostelium* require the other components of the γ-secretase complex (McMains et al., 2010). Our data thus provide evidence for a highly conserved structural role for presenilin proteins in development.

The conserved cellular role for human presenilin 1 that we have shown in *Dictyostelium* will enable a more thorough analysis of the cellular function of the wild-type protein in isogenic cell lines. Multiple gram weights of identical cells can be employed for biochemical analysis in this model, and a range of genetic approaches [including suppression screens and pharmocogenetics (Williams, 2005)] can be employed to better understand wild-type presenilin function in cells. *Dictyostelium* can also be used to analyse the >170 presenilin mutations that give rise to FAD (Parks and Curtis, 2007) in relation to basic cellular functions (such as calcium regulation) and in relation to γ-secretase activity [we can monitor the cleavage of human APP in *Dictyostelium*, giving rise to a potential change in the Aβ40:Aβ42 ratio associated with Alzheimer's disease progression (McMains et al., 2010)]. This new presenilin model could provide an important versatile system for future research on FAD and drug-development studies in translational research.

## MATERIALS AND METHODS

### Phylogenetic and structural analysis

MEGA4 was employed to align protein sequence data (Kumar et al., 2008), with further edits and the removal of highly variable regions conducted in Se-ALv2.0 (Rambaut, 1996). The phylogenetic relationships within this dataset were estimated by Bayesian analyses, using human signal-peptide-peptidase-like-2A (SPLLA2; Q8TCT8) as the outgroup, because presenilins and this protein belong to different subgroups of the peptidase A22 family (A22A and A22B, respectively). Bayesian trees were constructed in MrBayes 3.2 (Ronquist and Huelsenbeck, 2003) with gamma distribution, proportion of invariable sites and implementation of the 'aamodelpr=mixed prior' that allowed selection for the optimum substitution model. Two independent runs using four chains (three heated, one cold) were run for $1\times10^{6}$ generations, with sampling every $1\times10^{3}$ generations and a burn-in period of 250 trees. Nodal support was determined by approximate posterior probabilities performed in MrBayes. Stabilisation and convergence between runs was assessed using Tracer 1.5 (Rambaut and Drummond, 2009). Protein structural analysis was based upon the human protein structure (Parks and Curtis, 2007).

### Presenilin-mutant cell lines and cell-type-differentiation analysis

Presenilin- and nicastrin-null mutants were generated by homologous integration using the Cre-Lox system (Faix et al., 2004). Wild-type *psenB* and human *psen1* (isoform 2) full-length cDNAs were ligated into pDM317 and pDM448 N-terminal-GFP expression constructs under control of the *act15* promoter (Veltman et al., 2009). Wild-type human *psen2* (isoform 1) with a *D. discoideum* codon bias was synthesised by MWG-Biotech (Germany) and ligated into the pDM448 N-terminal-GFP expression construct. Stalk-cell development was conducted as described previously (Williams et al., 1999). Spore production was assessed by the lysis of mature fruiting bodies (24 h) in 0.1% NP40 or 0.3% Triton X-100 and counting spores with a haemocytometer.

### cAMP assays

Cells were grown in HL-5 medium containing selection antibiotics where appropriate. Cells were developed on non-nutrient (NN) agar (0.68 g/l $KH_2PO_4$, 0.89 g/l $Na_2HPO_4$•$2H_2O$ pH 6.5), 15 g/l agar], and samples

were harvested in PB (0.68 g/l $KH_2PO_4$, 0.89 g/l $Na_2HPO_4•2H_2O$ pH 6.5) at 0, 4 and 8 h of development. $5\times10^7$ cells were resuspended in 200 µl of PB and were lysed with 300 µl 3.5% (v/v) $HClO_4$. The lysates were neutralised by the addition of an equal volume of 50% saturated $KHCO_3$ and 200 µl of cAMP-assay buffer (4 mM EDTA in 150 mM sodium phosphate pH 7.5). The lysates were centrifuged for 5 min at 3000 ***g*** to precipitate protein and $KClO_4$. cAMP was assayed in 20 µl and 40 µl of the supernatant fraction by isotope-dilution assay, using purified protein kinase A regulatory subunit (PKA-R) from beef muscle as a cAMP-binding protein and [2,8-$^3$H]cAMP as the competitor. The data represent the mean and s.e.m. of five experiments performed in triplicate. The data from individual measurements were not normally distributed and significant differences between datasets were therefore estimated by Kruskal–Wallis ANOVA on ranks.

### Calcium assays

Calcium homeostasis in *Dictyostelium* cells was measured by using the calcium-sensitive aequorin approach in which strains were transformed with an apoaequorin-expressing plasmid (pPROF120) that allows real-time assay of cytosolic free $Ca^{2+}$ levels (Allan and Fisher, 2009). Briefly, $10^8$ cells were incubated in 5 ml of MES development buffer [10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.2), 10 mM KCl, 0.25 mM $CaCl_2$] containing coelenterazine-*h* (0.5 µg/ml dissolved in 20% w/v Pluronic F-127) for 7 h in shaking suspension, followed by washing with MES development buffer to remove residual coelenterazine-*h*. The coelenterazine-*h* allows *in vivo* reconstitution of the functional photoprotein, which upon calcium binding produces luminescence that can be detected with a photometer. In order to measure calcium influx upon cAMP stimulation, the total possible light emission was determined to normalise the aequorin luminescence signals. These values allowed calculation of an *in vitro* calcium concentration-effect curve upon 1 µM cAMP stimulation. All measurements were carried out in a New Brunswick ATP Photometer, as described previously (Allan and Fisher, 2009), and analysed using the R statistical package (R Core Team, 2012, http://www.R-project.org/).

### Immunofluorescence and western analysis

Monoclonal antibodies against calnexin were kindly provided by Annette Müller-Taubenberger (Müller-Taubenberger et al., 2001). For immunofluorescence labelling, cell lines expressing the *D. discoideum* or human genes were grown overnight in low-fluorescence medium, fixed, probed with anti-calnexin antibodies and labelled with goat anti-mouse-IgG (NEB 4409s; Alexa Fluor 555; Ipswich, MA). Rabbit monoclonal anti-presenilin-1 D39D1 antibody (#5643) was obtained from Cell Signaling Technology (Danvers, MA) and anti-presenilin-2 (EP1515Y) antibody (ab51249) was obtained from Abcam (Cambridge, MA) and used at 1:1000 for western analysis.

### Quantitative RT-PCR

Total RNA was extracted from *Dictyostelium* using the High Pure RNA Isolation kit (11828665001; Roche; Welwyn Garden City, UK) and cDNA was amplified using the First Strand cDNA Synthesis Kit (K1612; Fermentas; Loughborough, UK). Real-time amplification with SYBR Green (Sigma, S4438) was performed in a Rotor-Gene 6000 (Qiagen Ltd, Manchester, UK). Triplicate samples were collected at each time point with two RT-PCR technical replicates and the level of transcription was quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). PCR primer pairs were designed using Primer3 (Rozen and Skaletsky, 2000).

### Notch cleavage assay

An S2-cleaved form of human Notch 1 that requires γ-secretase-dependent S3 cleavage was used in a CBF1-reporter-gene assay as described previously (Hooper et al., 2006). The CBF1 reporter contains four tandem consensus DNA-binding sites in the promoter driving luciferase expression (a gift fromGerry Weinmaster, UCLA Medical School, CA). Presenilin-dependent generation of the Notch intracellular domain is required to activate CBF1-dependent transcription. Full-length *Dictyostelium* presenilin was expressed from the pFLAG-CMV5a vector (Sigma-Aldrich St Louis, MO).

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### Competing interests

The authors declare no competing interests.

### Author contributions

The project was conceived and designed by R.S.B.W., with additional experimental design input by M.H.R.L. M.H.R.L. and G.P.O. performed the majority of the experiments. C.S. and Z.-H.C. performed the cAMP experiments. C.A. and M.H.R.L. performed the calcium experiments. R.K. and M.H.R.L. performed the mammalian experiments. S.B. performed the phylogenetic analysis. P.W.B., A.R.K., P.F. and R.K. contributed reagents, materials or analysis tools and provided manuscript comments. R.S.B.W., M.H.R.L. and G.P.O. wrote the paper. G.P.O. did not participate in experiments involving animals, or cells or tissues from animals or from human embryos.

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### Supplementary material

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